Prothrombin Time

Determination by a Whole Blood Micro-Method for Control of Anticoagulant Therapy

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• **A micro technique that is here described for "prothrombin time" determinations, employing capillary whole blood, provides a range of values which is closely correlated with the Quick one-stage plasma method, thus providing interchangeability of results both in normal persons and in patients who have been treated with anticoagulant drugs.**

Avoidance of the use of a water bath and centrifuge permit this technique to yield immediate results at the bedside, in the office or in the patient's home.

The use of a whole blood instead of a plasma technique lends additional safety to control of anticoagulant medication, since it may reflect depression of clotting factors not apparent by the usual plasma methods.

PROPER USE of oral anticoagulants demands reliable laboratory control.[4] We shall describe a simple micro-method for testing whole blood "prothrombin time" or "activated clotting time." This method is applicable to clinical control of anticoagulant medication.

Ideally, laboratory tests to control anticoagulant medication should be devised so that:

1. *In vitro* conditions in the performance of the test should approximate as nearly as possible the *in vivo* conditions.

2. Results of any method should be readily reproducible and permit favorable comparison with other standard one-stage methods, in both absolute and relative aspects.

3. Only micro-quantities of capillary blood should be employed, thus avoiding the frequent venipuncture which may be required on a single patient.

4. Results should be available immediately for the guidance of the physician in regulation of anticoagulant dosage.

5. The interval between drawing blood and testing should be as short as possible to avoid erroneous results due to adverse effects of storage.

6. Performance of the test should be technically simple so that, in addition to laboratory personnel, physicians, nurses, or sometimes even patients themselves may achieve proficiency in the determination.

7. Employment of cumbersome equipment such as centrifuges and water baths should be eliminated so that tests may be done at bedside, in the patient's home or in the office of the physician responsible for anticoagulation administration.

Usually for the control of oral anticoagulant therapy as previously practiced, a plasma method is used, the end point of which is determined by recalcification of plasma in the presence of added thromboplastin. This is commonly designated as the one-stage "prothrombin time" or the Quick method.[14] As the therapeutic modality of anticoagulation has become more extensively employed, certain disadvantages, both practical and theoretical, have appeared in this time-honored technique. Among the disadvantages of the standard one-stage Quick method are:

1. Venipuncture is required. Usually 3 to 5 ml. of blood is taken.

2. Time for performance of this test, as usually dictated by custom in hospitals and clinical laboratories, is frequently as much as several hours after venipuncture. This time lag greatly lessens the usefulness of the test to the clinician, involving additional communication with the patient or the nurse.

3. Errors arise due to the effects of storage of citrated or oxalated blood. These sources of error include alteration of contact factor (Hageman factor),[9,11,19] labile factor (Factor V),[1,2] platelet Factor I, antihemophilic globulin (Factor VIII),[11] and antithrombin.[2]

4. Performance of the plasma test requires a centrifuge and a water bath, restricting its use to laboratories which often do not function during the entire 24 hours of the day.

5. There is considerable variability in the results from one laboratory to another, both with respect to normal and therapeutic ranges.[10]

6. Tests employing plasma have the theoretical disadvantage of being insensitive to possible deficiency of Factor IX (Christmas Factor, Antihemophilic Globulin, PTC).[9,12]

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The clinical need for a test giving immediately available results has led to various methods of employing mixtures of whole blood and thromboplastin to determine "activated clotting time" rather than plasma "prothrombin time."*

Whole blood methods that neglect control of critical temperatures have not yielded results comparable to those obtained by plasma methods in absolute terms of seconds elapsed. Other methods in which temperature control is included, such as one reported recently by Phillips and coworkers,[13] while giving excellent relative correlation with the Quick plasma method, fail to correspond in absolute values, especially in the higher ranges of anticoagulation. Such disparity demands radical revision of the clinician's concept of a safe therapeutic range, making for confusion when compared with standard Quick determinations.

Hoffman and Custer[7] in 1942 reported a micro-method at 37° C. for determination of "prothrombin time" on fresh capillary blood. Since physical conditions were controlled as in the original Quick procedure, their values were comparable both in absolute and in relative terms. They observed that variation in hematocrit caused no divergence in comparing plasma with capillary whole blood techniques. By dilution techniques their micro-method showed ". . . a parallelism with the Quick values usually within 5 per cent prothrombin."

The essential requirements of accurate temperature control, elimination of water bath and centrifuge, use of fingertip blood, and convenient portability seem to have been satisfied in the method herein reported.

MATERIALS AND METHOD

Instrument

A portable instrument‡ (14x15x9.5 cm.) provides a constant temperature for performance of the test. A built-in timing device composed of a small synchronous motor and a counter calibrated to 0.1 seconds is used to measure the observed clotting time.

A soft glass capillary tube (1.2-1.4x75 mm.) is employed to draw up the blood and the thromboplastin. A piece of soft rubber tubing, as used for standard blood-diluting pipettes, is equipped with a plastic mouthpiece at each end. A small rubber bulb similar to those used with smallpox vaccine tubes is stretched over the end of one of the plastic mouthpieces. The capillary tube is inserted into the other end of the vaccine bulb.

The clotting reaction takes place in the dimple of a glass depression slide which rests on a heated aluminum block, the temperature of which is thermostatically controlled at 38.5° C. Measurement of the surface temperature of the glass slide varies from 37° to 38° C., depending upon the ambient temperature and convection currents in the air.

Thromboplastin

Commercially available rabbit brain-lung thromboplastin† was employed. When using capillary blood the presence or absence of added calcium ions in the commercial thromboplastin mixture is immaterial, since the patient's own whole blood calcium is sufficient for the clotting action *in vitro*. If, for some reason, oxalated or citrated venous blood is tested, the thromboplastin mixture must contain added calcium chloride.

Method

1. After both the instrument and the thromboplastin reagent have attained the optimum temperature of 37° C., the finger or ear lobe is punctured cleanly and deeply enough to insure a free flow of blood without excessive squeezing. Whole venous blood may be used, provided the optimal ratio of citrate or oxalate is employed.

2. The first drop of blood is wiped away and the fingertip or ear lobe is gently squeezed to produce a large fresh drop of capillary blood.

3. A soft glass capillary tube is employed to draw up the blood to about one third of its capacity. The tip of the capillary tube is then wiped clean to avoid contamination of the thromboplastin reagent, and the blood is drawn 2 or 3 mm. farther up into the tube. This aids in preventing premature mixing of the blood and thromboplastin within the tube.

4. Without delay, an amount of thromboplastin approximately equal to the volume of blood in the tube is drawn up and the contents of the tube are then expelled into the dimple of the warmed glass depression slide. The timer is started.

5. With a clean metal "clot hook" (a scleral retractor is suitable), the blood-thromboplastin mixture is stirred in a rapid rotary fashion. The timer is stopped when gross clotting is observed. With normal blood and also with blood from patients receiving anticoagulants the entire mixture appears to clot simultaneously.

PRECAUTIONS

Accuracy and close agreement of results are dependent on absolute adherence to obvious, but easily overlooked, manipulations. Among these are:

*References 7, 8, 15, 16, 17, 18.

‡Prothrometer® manufactured by Oxford Laboratories, Redwood City, California.

†Simplastin® manufactured by Warner-Chilcott Co., Morris Plains, New Jersey.

1. Enough time should be allowed for the heating block of the instrument and the thromboplastin reagent to attain optimum temperature before proceeding with the test—usually about 10 to 15 minutes if the machine has been at room temperature.

2. The thromboplastin reagent should be fresh and should not have been contaminated by blood from previous determinations. When possible, the consistent use of a single commercial source of thromboplastin helps to insure uniformity of results.

3. Free-flow of capillary blood is essential. Excessive squeezing of the finger or earlobe will introduce tissue fluids which may alter the test. If more than two determinations are to be made at one time, another area should be pricked.

4. The ratio of the capillary blood and the thromboplastin reagent should be close to 1:1. Premarking of the capillary tubes is helpful in this measurement.

5. Blood should be drawn into the capillary tube before the thromboplastin. Reversal of this sequence increases the likelihood of premature mixing of the reactants due to differences in their relative viscosities.

6. In doing duplicate determinations, time may be saved by not resetting the timer and merely making a mental note of the initial determination.

RESULTS

Using the procedure outlined above, the "prothrombin time" or "activated clotting time" on probable normals lay in the range of 10 to 13 seconds, thus comparing favorably with results of the Quick method. It has been our practice to report results in seconds rather than as a "per cent of normal." In order to establish the viability of the thromboplastin, a "normal" was determined by random sampling or by utilizing commercial pooled plasma. Thromboplastins that yielded a "normal" value greater than 14 seconds were not used.

In establishing a comparison of the whole-blood microcapillary method with the standard one-stage plasma test of prothrombin time, every effort was made to maintain ideal test conditions. Dilution of venous blood and citrate was made with volumetric precision, employing graduated centrifuge tubes. When difficulty with venipuncture necessitated several attempts, the samples were discarded. The citrated venous blood was centrifuged immediately for 5 minutes at 1,600 r.p.m. and the plasma layer removed at once. In all instances the macro-method determination was performed within 15 to 20 minutes following venipuncture. When the micro-technique was applied both to finger-puncture capillary blood and to freshly drawn citrated venous blood, close agreement of results prevailed. Progressive shortening of capillary blood prothrombin time was noted following second or third determinations obtained from a single puncture site, thus underscoring the necessity of fresh, free-flowing capillary blood for accurate analysis.

The same vial of lyophilized thromboplastin was used for both methods in each comparative determination, and it was not used more than 48 hours after reconstitution even though it was preserved at 5° C.

The data that were analyzed consisted of 205 pairs of observations obtained by the whole blood micro-method and the plasma macro-method (Quick), divided for statistical purposes into groups "A" and "B" consisting of 182 and 23 pairs respectively.

In group "A" (182 pairs) each observation on the micro- or macro-methods was the average of two determinations. Group "B" (23 pairs) included 20 pairs of observations from single, duplicate or triplicate determinations and 3 pairs where the whole blood micro-method gave an unknown reading greater than 50 seconds. All comparative results are combined on the scattergram shown in Chart 1.

For group "A" (182 pairs) the estimated regression line is

$$y = .08 + .96x,$$

where x and y represent the prothrombin times for the micro- and macro-methods, respectively, and the correlation coefficient (r) between x and y is $r = +.96$. The statistical analysis* established a definite linear relationship between the methods, even though it is not the ideal direct relationship $y = x$. For prothrombin times up to 50 seconds, the estimated regression line lies significantly (at the 1 per cent level) below the line $y = x$; that is, the micro-method tends to give a slightly higher prothrombin time than the macro-method. However, at the point of maximum difference, namely, at a prothrombin time of 50 seconds, the average statistical discrepancy between the two methods is just 2 seconds.

In calculating the statistical correlation, three instances involving two patients were omitted. In these three instances the microcapillary method produced results much longer than the macroplasma method. The macro-method showed values of 33.0, 20.6, and 39.6 seconds, as compared with microcapillary figures in excess of 50 seconds. We omitted these three comparative tests because prolongation of micro-test results apparently reflected the diminution of coagulation factors other than those in the *extrinsic* coagulation system. It is generally agreed that drugs of the coumadin type act to depress both the *intrin-*

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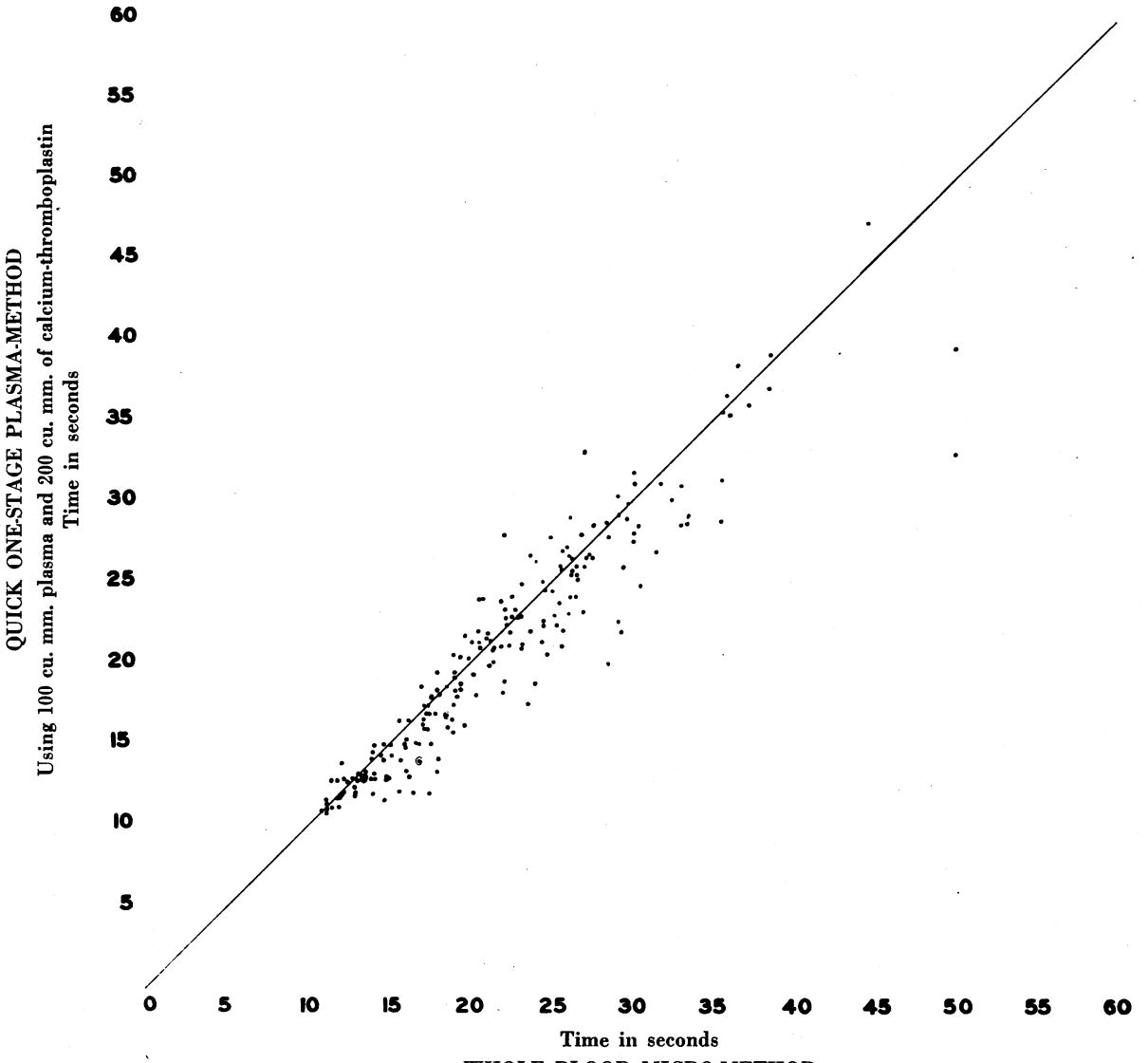

Chart 1.—Comparison of "prothrombin time" determinations by the Quick one-stage plasma method and the whole blood micro-method.

sic and the *extrinsic* clotting systems.† Prothrombin (Factor II) and Stuart-Prower factor (Factor X) are affected in both systems, as is Christmas factor (Factor IX) in the *intrinsic* system and proconvertin or stable factor (Factor VII) in the *extrinsic* system.[12] The one-stage plasma (Quick) test is not sensitive to depression of Factor IX but reflects alteration of activated clotting time associated only with the *extrinsic* system.

Two of the three test pairs showing longer micro-capillary than macroplasma prothrombin times occurred in one subject, a 79-year-old man with hepatic cirrhosis and gross hematuria. While the plasma macro-method gave figures (33.0 and 20.6 seconds) in the "therapeutic range of anticoagulation" his blood failed to clot at 50 seconds by the micro-capillary method. The third widely divergent comparison occurred in a 69-year-old arteriosclerotic diabetic patient who had pronounced gingival bleeding while his macroplasma prothrombin time was 39.6 seconds and clotting did not occur at 50

†Clotting factors present in circulating blood are designated *intrinsic*. Activation of the *extrinsic* system requires tissue thromboplastin. In the formation of thrombin by the *intrinsic* system the following factors are required: Factor II (prothrombin), Factor IV (calcium), Factor V (proaccelerin), Factor VIII (antihemophilic globulin), Factor IX (Christmas factor, plasma thromboplastin component, or antihemophilic B factor), Factor X (Stuart-Prower factor), Hageman factor, platelets ("cephalin"), and plasma thromboplastin antecedent. The *extrinsic* system requires Factors II, IV, V, VII, and X plus Factor III (tissue thromboplastin). Oral anticoagulants depress Factors II, VII, IX, and X.[12]

seconds by the microcapillary method. The discrepancies in these three instances cannot be explained by technical error. The presence of spontaneous bleeding while the "prothrombin time" done by the Quick method was in the "therapeutic" range would indicate that there was a diminution in a coagulation factor to which the conventional plasma method was insensitive. Exact delineation of the additional treatment-induced clotting defect would have required more selective methods—the thromboplastin generation test, for example.

DISCUSSION

In the use of anticoagulant therapy, patients with defects in accessory clotting factors occasionally will be encountered. Neither the technique described in this paper nor the one-stage plasma "prothrombin time" may be considered a diagnostic tool in such problems. These one-stage tests measure only the ability of blood to clot within a given time when exposed to excess tissue thromboplastin and calcium ion. Prothrombin may not be specifically assayed by one-stage techniques. A more specific assay of prothrombin concentration is possible by two-stage techniques or by hydrolysis of a selective substrate, as in the TAME[6] method. Results are still expressed, however, in terms of clotting activity rather than a quantitative absolute value; furthermore, the technical difficulty involved in the more elaborate tests makes reproducibility troublesome, from one laboratory to another. Apart from its technical difficulty the TAME procedure is unsuited to routine control of anticoagulant medication, since it does not reflect depression of other blood clotting factors which are influenced by oral anticoagulants.

The one-stage "prothrombin" test, whether performed on plasma or whole blood, does not reveal defects in thromboplastin formation. Additionally, the blood of a hypofibrinogenemic patient will have a prolonged one-stage "prothrombin time" in the presence of normal plasma concentration of prothrombin.[2] In spite of these limitations, the one-stage tests are clinically accepted as guides to oral anticoagulant dosage. There are, however, three areas in which a rapid method for measuring "prothrombin" time of capillary blood appears to offer a distinct increase in safety and accuracy over the conventional plasma test. These situations involve the elimination of innate sources of error associated with: (1) Storage of plasma or blood, (2) repeated venipuncture, (3) disturbance of critical calcium ion concentration essential for accuracy in the one-stage plasma technique.

1. Since the microcapillary whole blood test is completed within seconds after initiating bleeding, the possibility of introducing additional coagulation factors, either acceleratory or inhibitory, due to storage of the blood, is largely eliminated. It is not uncommon for at least a half to three-quarters of an hour to elapse between venipuncture and centrifugation of the specimen. Frequently as much as several additional hours of storage intervene before the daily determination of "prothrombin times" is completed in a given laboratory. Delays in testing are owing in part to the convenience of waiting for a backlog of tests to accumulate so that a number can be done at one time, and partly to lack of cognizance by laboratory personnel of the various factors that may drastically alter the one-stage test. Chief among these storing phenomena are:

(a) *Labile Factor* (Factor V, proaccelerin) is essential for the conversion of prothrombin to thrombin, and its diminution appears to be solely a consequence of storage.[2] Storage does not impair the actual prothrombin concentration, but the one-stage "prothrombin time" progressively lengthens as the plasma ages. Thus, in anticoagulated patients, the unrecognized diminution of labile factor may lead to incorrect interpretation on the part of the clinician of the degree of anticoagulation achieved.

(b) Acceleration of clotting time, as measured by one-stage methods, becomes apparent within two hours, due to presence of an "activation substance" produced by interaction between Hageman factor (contact factor) and plasma thromboplastin antecedent.[12] Hageman factor also serves to activate Factor VII in the *extrinsic* clotting system, even in the absence of calcium[19] and at refrigeration temperatures,[11] so that one may assume its influence to be undiminished in refrigerated plasma. Comparative trials with plain glass and siliconized capillary tubes in the whole blood micro-method indicated that the period of contact between glass and blood is so brief as to be of negligible concern in this technique.

To avoid the influence of contact factor when glass tubes are used for collection of venous blood, Owren stressed that normal blood should be tested within a few minutes and blood from anticoagulated patients within one hour after venipuncture.[11] Assurance of such prompt disposition of the procedure cannot be gained from most laboratories doing the plasma one-stage technique.

(c) *Platelet Factor I* (platelet accelerator) acts to accelerate the conversion of prothrombin to thrombin in stored plasma, and may result in spurious shortening of the "prothrombin time."[5]

2. In dispensing with the necessity for large amounts of venous blood, one removes the possibility of partial undetected coagulation which may commence in the syringe when venipuncture is difficult. Serum produced by this premature coagulation elicits an accelerator substance which promotes the conversion of prothrombin to thrombin during

the test procedure,[2] again making an erroneously short "prothrombin" time.

3. Critical control of calcium ion concentration demanded by usual plasma "prothrombin time" methods is not a factor in the capillary blood technique where the physiological concentration of calcium remains undisturbed. The artificial situation in which blood calcium is removed by oxalate or citrate during venipuncture and then replaced when the "prothrombin time" is determined lends itself to technical errors which would profoundly affect the result. Excess *in vitro* anticoagulant, which may be present when the ratio of blood to balanced oxalate is less than 9:1, continues to precipitate the calcium ion added during the prothrombin testing procedure and makes for an excessively prolonged "prothrombin time," and sometimes a clot does not appear at all. The existence of excess oxalate may also be encountered when the hematocrit is higher than normal, requiring an increased concentration of calcium ion in the test procedure. In the microcapillary technique with whole blood, it appears that only an excess of calcium ion is required, since little difference in results was noted whether the material used was a commercial thromboplastin combined with calcium chloride* or a product in which the calcium was supplied separately and could be used as thromboplastin alone.†

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